Carcinogenesis vol.20 no.11 pp.2159–2165, 1999

Strain-dependent lung tumor formation in mice transplacentally exposed to 3-methylcholanthrene and post-natally exposed to butylated hydroxytoluene

Kiersten M.Gressani[1,2], Sandra Leone-Kabler[2], M.Gerard O'Sullivan[4], L.Douglas Case[3], Alvin M.Malkinson[5] and Mark Steven Miller[1,2,6]

[1]Department of Physiology and Pharmacology, [2]Department of Cancer Biology and [3]Department of Public Health Sciences, Comprehensive Cancer Center, Wake Forest University School of Medicine, Winston-Salem, NC 27157, [4]Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Minnesota, St Paul, MN 55108 and [5]Department of Pharmaceutical Sciences, School of Pharmacy, Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA

[6]To whom correspondence should be addressed at: Department of Cancer Biology, Wake Forest University School of Medicine, Medical Center Boulevarde, Winston-Salem, NC 27157, USA
Email: msmiller@wfubmc.edu.

The carcinogenic effects of *in utero* exposure to 3-methylcholanthrene (MC) have been demonstrated in the tumor-resistant C57BL/6 (B6) and DBA (D2) strains of mice. In this study, we determined the effects of *in utero* exposure to MC in BALB/c mice, a strain which demonstrates greater susceptibility to lung tumor induction, and compared our findings with those previously found in $[D2 \times B6D2F_1]F_2$ mice. In addition, we assessed the molecular pathogenesis of the chemically induced tumors and examined the effects of the putative lung tumor promoter butylated hydroxytoluene (BHT) in BALB/c mice. BALB/c mice were treated on day 17 of gestation with 5, 15 or 45 mg/kg MC and 6 weeks after birth with BHT for 6 consecutive weeks. Mice were killed at 6 months of age. Ki-*ras*, $p16^{Ink4a}$ and $p19^{ARF}$ gene loci were amplified from paraffin-embedded lung tumor tissue and screened for the presence of point mutations via allele-specific oligonucleotide hybridization and single strand conformation polymorphism (SSCP) analyses. Ki-*ras* point mutations were found in 56% (20/36) of BALB/c lung tumors, with 33% (2/6) of the hyperplasias, 58% (10/19) of the adenomas and 73% (8/11) of the carcinomas exhibiting point mutations at this gene locus. Similar incidences of Ki-*ras* mutations were previously found following transplacental exposure of $[D2 \times B6D2F_1]F_2$ mice to MC and treatment of adult A/J mice with urethane. Interestingly, a strain-dependent difference was observed in the mutational spectrum. Sixty-two and 38% of the lung lesions in BALB/c mice exhibited G→C and G→T transversions, respectively, in contrast to the 13 and 84% incidences previously observed in $[D2 \times B6D2F_1]F_2$ mice. SSCP analysis of the tumor suppressor gene $p16^{Ink4a}$ showed a 6% incidence of point mutations, consistent with that found in $[D2 \times B6D2F_1]F_2$ mice. No mutations were found in exon 1β of the $p19^{ARF}$ gene of either strain. BHT, a lung tumor promoter in adult mice, had no statistically significant effects on either tumor incidence, tumor multiplicity or the mutational spectrum produced in the Ki-*ras* gene by *in utero* MC treatment. However, though not significant, there was an observable trend in increased tumor multiplicity in mice co-treated with BHT. These data demonstrate the transplacental carcinogenic effect of MC in BALB/c mice and show that mutagenic damage to Ki-*ras* is a critical early event mediating murine lung tumorigenesis in both the tumor-sensitive and tumor-resistant strains. Unlike what occurs when adult BALB/c mice are treated with MC, BHT does not appear to significantly promote the formation of lung tumors following transplacental exposure to MC, possibly due to the rapid growth and cell proliferation in the developing organism. Strain-dependent differences in the Ki-*ras* mutational spectrum may be associated with their differential susceptibility to lung tumor initiation.

Abbreviations: ASO, allele-specific oligonucleotide hybridization; BHT, butylated hydroxytoluene; CYP1A1, cytochrome P4501A1; MC, 3-methylcholanthrene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PAH, polycyclic aromatic hydrocarbon; SSCP, single strand conformation polymorphism.

Introduction

The developing fetus is highly sensitive to the effects of *in utero* exposure to environmental toxicants, such as those found in cigarette smoke and in other forms of air pollution (1–3). Oxidative metabolism of these agents by both the mother and fetus causes DNA damage in tissues of transplacentally exposed fetuses, as well as multiple developmental impairments (4,5). Prenatal exposure to similar compounds also increases the risk of adult and childhood cancers (6,7). Polycyclic aromatic hydrocarbons (PAHs), present in tobacco smoke, charbroiled foods and air pollution, are potent carcinogens (8). PAHs readily cross the placental membrane to induce fetal cytochrome P4501A1 (CYP1A1), which converts these compounds into DNA-damaging molecules (9,10). A strong association is seen between induction of CYP1A1 and levels of PAH–DNA adducts in human cord blood and placental tissues (11,12) and rodent lung and liver tissue (13). This appears to correlate with the incidence of transplacentally induced lung tumors (2,3,14,15).

Tumor formation is a multistep process in which the loss of growth control is an early step, with cell cycle regulatory genes playing important roles in this process. Mutational activation of the proto-oncogene *ras* occurs in ~30% of all human tumors (16). Alterations in the Ki-*ras* isoform are particularly prevalent in lung neoplasms (17,18). Mutational inactivation of tumor suppressor genes, such as $p16^{Ink4a}$ and *p53*, is also frequently observed (19–21). These genes behave primarily as negative regulators of cellular proliferation, hence, when mutated the cell loses a critical 'brake' on cell growth which can ultimately result in the formation of tumors (22). $p16^{Ink4a}$ belongs to the *Rb*/cyclin D1/$p16^{Ink4a}$ regulatory pathway. As a G_1-specific cell cycle regulatory gene, $p16^{Ink4a}$ blocks progression of the cell cycle by binding to cyclin-dependent kinases 4 and/or 6 to prevent formation of active complexes with cyclin D1, thereby preventing phosphorylation and inactivation of pRB protein (23). Hence, loss of $p16^{Ink4a}$

can result in unregulated cellular proliferation. Likewise, inactivation of the *p53* gene, which is involved in G_1 arrest of the cell cycle and the initiation of apoptosis, can lead to unchecked cell growth in the presence of damaged DNA (21). The role of proto-oncogenes and tumor suppressor genes in tumorigenesis in the adult model has been well studied, however, little is known about the molecular alterations in these cell regulatory genes in transplacentally induced neoplasia.

The induction of tumors following *in utero* exposure to 3-methylcholanthrene (MC) has previously been demonstrated in the relatively tumor-resistant C57BL/6 (B6) and DBA/2 (D2) strains of mice (14,24), but not in the more sensitive BALB/c mice. Differences in lung tumor susceptibility between these strains has been ascribed to several genes (25). A polymorphism in the second intron of Ki-*ras* plays a major role in lung tumor susceptibility, as determined using carcinogenesis protocols in adult mice (25,26). The Ki-*ras* allele found in susceptible BALB/c mice contains a 37 bp deletion in the second intron compared with the resistant B6 and D2 strains (26). What effect this polymorphism has on the response of these strains to transplacental carcinogenesis has yet to be determined. Sequence polymorphisms have also been identified in the murine $p16^{Ink4a}$ tumor suppressor gene at nucleotides 53 and 61 of exon 1α and at nucleotide 33 of exon 1β in BALB/c and other strains of mice (27). The utilization and comparison of the tumorigenic response of different strains could provide important information regarding these noted polymorphisms. Further, assessing the effects of these polymorphisms on transplacental carcinogenesis is warranted in considering the validity of chemical induction of tumorigenesis for screening purposes.

Butylated hydroxytoluene (BHT), a commonly used dietary preservative, is a lung tumor promoting agent. MC treatment followed by chronic exposure to BHT results in a vast increase in tumor number above that found with the MC initiator alone in the adult (28). Little is known about the effects of prenatal exposure to MC followed by post-natal exposure to BHT. BHT acts as a promotor in several organ systems other than the lung, such as the liver, bladder, stomach and colon (29). Postulated mechanisms by which BHT exerts its tumor promoting effects are analogous to those hypothesized for other promoting agents, such as phorbol esters (30), which include the following: BHT induces a chronic inflammatory response (31); BHT interferes with gap junctional intercellular communication (32); BHT preferentially induces apoptosis in normal cells (33).

The serious risks associated with *in utero* exposure to environmental carcinogens necessitate the need for further study in this area. The present study is part of our ongoing efforts to assess environmental/genetic interactions relating to *in utero* exposure to PAH. We demonstrate herein strain differences in the effects of MC on offspring from different strains of mice, on the mutational spectrum of MC-induced tumors and on the effects of BHT on tumor yield.

Materials and methods

Chemicals

BHT, MC and olive oil were purchased from Sigma Chemical Co. (St Louis, MO); Denhardt's solution and dextran sulfate from 5 Prime→3 Prime (Paoli, PA); formamide from Fluka Chemical Corp. (Hauppauge, NY); MDE gel solution from FMC Corp. (Rockland, ME); [γ-^{32}P]ATP (~6000 Ci/mmol) from DuPont/NEN (Boston, MA); Tween 20 from Aldrich Chemical Co. (Milwaukee, WI).

Table I. Oligonucleotide primers used for PCR amplification

Ki-*ras*	exon 1	5′ primer	5′-ATGACTGAGTATAAACTTGT-3′
		3′ primer	5′-TCGTACTCATCCACAAAGTG-3′
	exon 2	5′ primer	5′-TACAGGAAACAAGTAATTGATGGAG-AA-3′
		3′ primer	5′-ATAATGGTGAATATCTTCAAATGATTT-AGT-3′
$p16^{Ink4a}$	exon 1α	5′ primer	5′-GGTCACACGACTGGGCGATT-3′
		3′ primer	5′-GAATCGGGGTACGACCGAAA-3′
	exon 2	5′ primer	5′-GTGATGATGATGGGCAACGT-3′
		3′ primer	5′-GGGCGTGCTTGAGCTGAAGC-3′
$p19^{ARF}$	exon 1β	5′ primer	5′-GCATGGGTCGCAGGGTTCTTGG-3′
		3′ primer	5′-CCTGGTCCAGGATTCCGGTGC-3′

Amplification conditions are listed in Materials and methods.

Animals and treatment protocol

Paraffin-embedded $[D2\times B6D2F_1]F_2$ lung tissue was generated as described in a previous study (24). $B6D2F_1$ male mice, heterozygous for the aryl hydrocarbon receptor gene and exhibiting the PAH-inducible, or responsive, CYP1A1 phenotype, were mated with non-responsive D2 females. This resulted in a litter in which half the fetuses had the responsive phenotype and half the non-responsive phenotype. On day 17 of gestation (day 1 being the day the vaginal plug was first detected), pregnant females were treated with a single i.p. injection of either 0.5 ml/35 g olive oil or 30 mg/kg MC dissolved in olive oil. Three days later, the $[D2\times B6D2F_1]F_2$ offspring in this study were born and foster nursed by untreated B6 or D2 mothers to avoid any further carcinogen exposure via the mother's milk. The mice were housed for 1 year and killed by cervical dislocation. In the current study, BALB/c mice were treated on day 17 of gestation with a single i.p. injection of either corn oil or 5, 15 or 45 mg/kg MC. Starting at 6 weeks after birth, these mice were given 6 consecutive weekly i.p. injections of 200 mg/kg BHT dissolved in corn oil or 100 μl of the corn oil vehicle. At 6 months of age the mice were killed by CO_2 asphyxiation.

Macroscopically visible lung tumors in all groups of mice were enumerated, removed, fixed and embedded in paraffin for later analyses. Hematoxylin and eosin stained lung sections were examined by a board certified veterinary pathologist, using standard histopathological criteria (34), to histologically classify lung lesions as hyperplasias, adenomas or carcinomas.

Extraction and amplification of DNA from paraffin-embedded lung tissue

Thirty-six randomly chosen paraffin-embedded lung tumor tissue samples from 25 MC ± BHT-treated BALB/c mice were cut from paraffin blocks with clean razor blades. The sections were deparaffinized and digested as previously described (35). A 2 μl aliquot of this lysate was subjected to PCR using the Perkin-Elmer GeneAmp PCR Reagent Kit. All reactions were carried out in 100 μl and consisted of reaction buffer (10 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 50 mM KCl), 200 μM dNTPs (dATP, dCTP, dGTP and dTTP) and 2 U of Ampli*Taq* Gold (Perkin Elmer). Primers for the Ki-*ras* (Oligos Etc., Guilford, CT), $p16^{Ink4a}$ and $p19^{ARF}$ genes (DNA Synthesis Core Laboratory, Comprehensive Cancer Center, Wake Forest University) were added at a final concentration of 0.2 μM for each primer (Table I). The samples were overlaid with 100 μl of mineral oil to prevent evaporation and cross-contamination of the samples. The PCR cycle parameters for exons 1 and 2 of the Ki-*ras* gene consisted of an initial 2 min denaturation step at 94°C, followed by 40 cycles of 1 min at 94°C, 2 min at 50°C and 2 min at 74°C, with a final extension step of 74°C for 7 min. For exons 1α and 2 of $p16^{Ink4a}$, an initial denaturation step of 94°C for 2 min was followed by 40 successive cycles of 1 min at 94°C, 2 min at 58°C and 2 min at 72°C, with a final extension step of 72°C for 7 min. To amplify exon 1β of $p19^{ARF}$, an initial denaturation step of 94°C for 2 min was followed by 40 successive cycles of 1 min at 94°C, 2 min at 67°C and 2 min at 72°C, with a final extension step of 72°C for 7 min. Each reaction included deparaffination procedure controls, which lacked tumor tissue but were mock extracted, and amplification negative controls, which lacked template DNA and served as negative buffer controls for the PCR amplification reactions. All samples were amplified in an Ericomp DeltaCycler II System.

Allele-specific oligonucleotide hybridization (ASO)

Thirty microliters of PCR products, diluted in 170 μl of sterile water, were heat denatured and blotted directly onto a Nytran membrane filter (Schleicher & Schuell, Keene, NH) using a Schleicher & Schuell minifold II slot blot apparatus. Amplified DNA products were fixed to the membrane by UV cross-linking and screened with 5′-end-labeled 20 bp oligonucleotides to mouse Ki-*ras* codons 12, 13 and 61 (Clontech, Palo Alto, CA). The filters were

prehybridized in oligonucleotide buffer at 37°C for 1.5 h. Following the addition of an oligomer 5′-end-labeled with ^{32}P (5×10^6 c.p.m./ml), the membrane was hybridized for 12–16 h and the filters washed under stringent conditions (3°C below the T_m); this allowed only fully matched probes to remain bound to DNA (36). The blots were visualized on a Molecular Dynamics PhosphorImager 445SI (Sunnyvale, CA).

Single strand conformation polymorphism analysis (SSCP) and sequence analysis

The mutational spectra of exon 1β of $p19^{ARF}$ of the BALB/c and $[D2\times B6D2F_1]F_2$ lung tumors and of exons 1α and 2 of $p16^{Ink4a}$ from BALB/c lung tumors were analyzed. Ten microliters of initial PCR products were added to 10 μl of stop buffer (95% formamide, 10 mM NaOH, 0.025% BPB, 0.025% xylene cyanol), heat denatured for 5 min at 95°C and chilled on ice. This mixture was electrophoresed on a non-denaturing 0.5× MDE gel in the presence or absence of 5% glycerol at 35 W for 3 h at 10°C ($p16^{Ink4a}$) or 30 W for 3.5 h at 10°C ($p19^{ARF}$) in 0.6× TBE running buffer on a Bio-Rad Universal Mutation Detection System apparatus (Bio-Rad, Hercules, CA). The gel was stained with ethidium bromide (10 mg/ml) for 30 min and visualized using UV light. DNA bands exhibiting a mobility shift due to possible mutations were excised for sequence analysis.

Each band was placed in 100 μl of nuclease-free water and heated to 80°C for 15 min. Thirty microliter aliquots were amplified as described above except that the number of cycles was reduced to 30. DNA was purified from low melting point agarose gels and cloned using the TA Cloning Kit (Invitrogen, San Diego, CA). Briefly, gel-purified products were ligated into pCRII cloning vectors and used to transform *Escherichia coli*. Plasmid DNA was isolated from transformed colonies using Qiagen columns (Qiagen, Chatsworth, CA) and sequenced using the ABI Prism sequencing kit. Ki-*ras* was sequenced directly from the initial PCR products. DNA sequence analysis was done using DNASIS software (Hitachi Software Engineering America, San Bruno, CA). To control for false positives, tumors were considered positive for mutations only when identical results were determined in a second independent experiment. All samples were sequenced in the forward and reverse directions to eliminate the possibility of polymerase-induced errors during sequencing.

Statistical analysis

Statistical analysis of the incidence and mutational spectra in lung tumors of $[D2\times B6D2F_1]F_2$ mice was previously reported (24,37). Briefly, χ^2 and Cochran–Mantel–Haenszel tests were performed to determine whether tumor stage and mutation type were independent of each other and whether there was an ordered association. In the present study, a χ^2 test was used to assess the significance of the difference in lung tumor incidence between the groups of BALB/c mice defined by their MC dose and BHT status. Logistical regression was then used to assess the joint effect of BHT and dose level and their interaction on tumor incidence. Rank analysis of covariance was used to assess the joint effects of BHT and dose level on tumor multiplicity. Models were run with and without an interaction between BHT and dose and with dose considered categorically and continuously. Separate models were run to assess the effect of BHT at each dose level. Fisher's exact test was used to assess the association between tumor stage and mutation type and between BHT and mutation type.

Results

In the present study, the role of genetic and environmental interactions on the initiation and pathogenesis of transplacentally induced lung tumors was investigated. BALB/c mice were treated with MC on day 17 of pregnancy and were subsequently given BHT 6 weeks after birth to assess the modulation of MC-induced lung tumors by BHT. Molecular alterations in the proto-oncogene Ki-*ras* and the tumor suppressor genes $p16^{Ink4a}$ and $p19^{ARF}$ were determined. Briefly, analyses of the genes were carried out with lysates from paraffin-embedded BALB/c lung tissue and compared with results obtained in previous studies on $[D2\times B6D2F_1]F_2$ offspring treated by the same protocol. The lysates were subjected to DNA amplification followed by ASO screening and SSCP analysis.

Gestational MC exposure at day 17 had no effect on maternal weight gain, length of pregnancy, birth weight, fetal mortality or post-natal weight gain (data not shown). BALB/c mice treated with vehicle or with the lowest dose of MC ± BHT (5 mg/kg) developed no macroscopically visible lung tumors; mice treated with higher doses had tumor incidences ranging from 20 to 100% (Table II). Dose-dependent increases in tumor incidence and multiplicity were statistically significant ($P < 0.05$, incidence; $P < 0.001$, multiplicity) in both BHT-treated and untreated groups. BHT co-treatment increased tumor multiplicity in the 15 and 45 mg/kg groups over that observed in mice treated with MC alone, however, the differences were only of borderline significance ($0.1 < P < 0.15$ at both doses). Despite the differences in susceptibility to lung tumorigenesis observed in adult mice (25), tumor incidences in the BALB/c mice were similar to those found in $[D2\times B6D2F_1]F_2$ mice (Table II). This may be related to the time course of the experimental protocols. $[D2\times B6D2F_1]F_2$ mice were killed following a 1 year bioassay, while BALB/c mice were killed following a 6 month bioassay. It is possible that the incidence of tumors in BALB/c mice would have been significantly greater than that found in $[D2\times B6D2F_1]F_2$ mice if the bioassay was carried out for a longer period of time. In addition, the resistant mice exhibit increased sensitivity to chemically induced lung tumor formation as a result of *in utero* exposure to chemical carcinogens (2,3). BALB/c mice may not demonstrate this increased sensitivity following transplacental exposures.

Table II. Lung tumor formation in transplacentally treated mice

	Tumor incidence (% mice with tumors)	Tumor multiplicity (no. tumors/mouse)
BALB/c mice[a]		
Corn oil	0/11, 0%	0
5 mg/kg MC + oil	0/11, 0%	0
5 mg/kg MC + BHT	0/7, 0%	0
15 mg/kg MC + oil	3/15, 20%	0.2 ± 0.4
15 mg/kg MC + BHT	6/13, 46%	0.8 ± 1.2
45 mg/kg MC + oil	11/11, 100%	2.6 ± 1.3
45 mg/kg MC + BHT	9/10, 90%	3.8 ± 2.2
$[D2\times B6D2F_1]F_2$ mice[b]		
Olive oil	0/26, 0%	0
10 mg/kg MC	3/27, 11%	0.2 ± 0.8
30 mg/kg MC	26/31, 84%	3.7 ± 3.2

[a]On day 17 of gestation, BALB/c mice were treated with a single i.p. injection of either corn oil (control group) or with 5, 15 or 45 mg/kg MC dissolved in corn oil. Six weeks after birth, mice were treated with weekly i.p. injections of either corn oil or 200 mg/kg BHT dissolved in corn oil for 6 consecutive weeks. At 6 months of age, the mice were killed by CO_2 asphyxiation and lung tumors were fixed in 10% formalin and embedded in paraffin. Tumor multiplicity is reported as means ± SD.
[b]On day17 of gestation, $[D2\times B6D2F_1]F_2$ mice were treated with a single i.p. injection of either olive oil (control group) or 10 or 30 mg/kg MC dissolved in olive oil. At 1 year of age, the mice were killed by cervical dislocation and lung tumors were fixed in 10% formalin and embedded in paraffin. Tumor multiplicity is reported as means ± SD. Tumor incidence data were previously reported by Wessner *et al.* (24).

Alterations in Ki-*ras* are frequently found in lung tumors of humans as well as experimental rodent models (16,17,38). Each Ki-*ras* codon (12,13,61) was screened using ASO with six different mutant 20 bp oligonucleotide probes differing from the wild-type sequence by 1 bp. Twenty of the 36 lesions (56%) excised from BALB/c mice exhibited mutations in exon 1 or 2, a lower percentage than that observed in $[D2\times B6D2F_1]F_2$ mice, where 37/47 lesions (79%) exhibited mutations in the first or second base of codons 12 or 13 ($P = 0.032$). Figure 1 shows the results of the ASO analysis

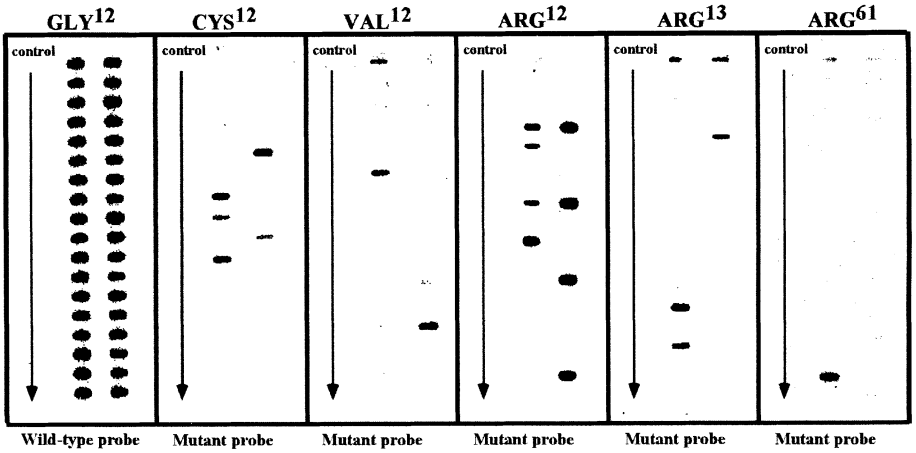

Fig. 1. ASO analysis of paraffin-embedded BALB/c lung tissue of the Ki-*ras* gene. The column labeled control includes procedure and negative controls. Each slot represents an individual tumor sample. The tumors were (from top to bottom): (middle) 1–18; (right) 19–36. The blot on the left was hybridized with a wild-type (Gly12) probe and the other five panels demonstrate sequential hybridization with different mutant probes. Data are representative of two independent experiments.

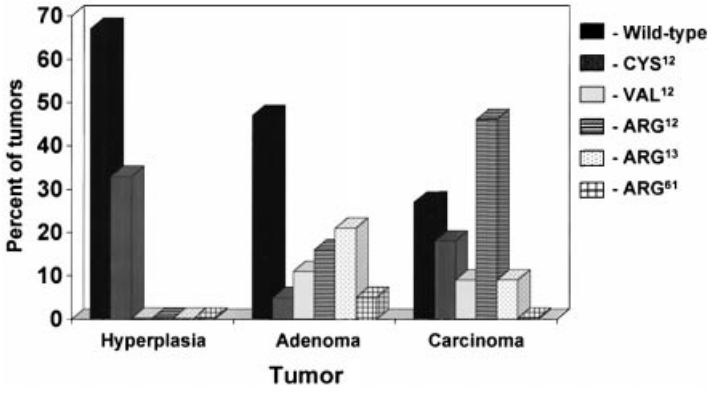

Fig. 2. Ki-*ras* mutational spectrum on the basis of tumor stage. Tumors were removed from BALB/c mice and were classified as hyperplasias, adenomas or carcinomas. The values represent the percentage of tumors of a specific histological classification harboring specific Ki-*ras* mutations. For example, 33% (2/6) of all the hyperplasias had a Cys12 transversion, while 67% (4/6) had the wild-type sequence. Note that one adenoma and one carcinoma contained two mutations.

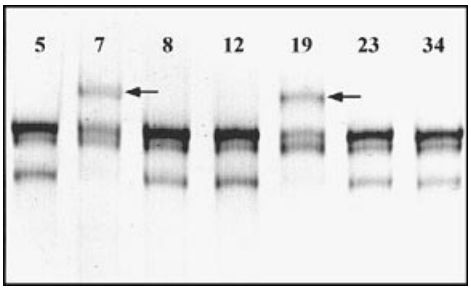

Fig. 3. SSCP analysis of BALB/c lung tissue of p16^{Ink4a} exon 2. A 10 µl aliquot from the primary PCR was added to 10 µl of stop buffer and heat denatured. The 20 µl mixture was electrophoresed in a non-denaturing 0.5× MDE gel at 10°C for 3 h at 35 W in 0.6× TBE running buffer. Tumor numbers are shown at the top of each lane. The arrows indicate band shifts in tumors 7 and 19. Data are representative of two independent experiments.

of the Ki-*ras* gene in lung tumor tissue of BALB/c mice. As shown in the left panel, all of the PCR products from normal and tumor tissue hybridized to the wild-type Gly12 probe. The other five panels demonstrate hybridization with mutant probes. Two separate amplification reactions and ASO analyses were performed with each sample to ensure that the results were not due to *Taq*-induced errors during primary amplification. Randomly chosen PCR products were sequenced to confirm the presence of these point mutations. All exon 1 Ki-*ras* mutations were at G residues; 38% of these were G→T transversions and 62% were G→C transversions. This is in contrast to that observed in the [D2×B6D2F$_1$]F$_2$ mice, where 84% of the Ki-*ras* mutations were G→T transversions and 13% were G→C transversions (37).

Of the hyperplasias, 67% (4/6) had the wild-type Ki-*ras* sequence and 33% (2/6) exhibited a GGT→TGT (Gly12→Cys12) transversion (Figure 2). Of the adenomas, 47% (9/19) had the wild-type sequence, 5% (1/19) exhibited a Cys12 (GGT→TGT) transversion, 11% (2/19) exhibited a Val12 (GGT→GTT) transversion, 16% (3/19) exhibited an Arg12 (GGT→CGT) mutation, 21% (4/19) exhibited an Arg13 (GGC→CGC) transversion and 5% (1/19) exhibited an Arg61 (CAA→CGA) mutation. Of the carcinomas, 27% (3/11) had a wild-type Ki-*ras* sequence, 18% (2/11) exhibited a Cys12 (GGT→TGT) transversion, 9% (1/11) exhibited a Val12 (GGT→GTT) transversion, 46% (5/11) exhibited an Arg12 (GGT→CGT) mutation and 9% (1/11) exhibited an Arg13 (GGC→CGC) transversion. None of the specific Ki-*ras* mutations differed significantly between tumor type. It should be noted that one adenoma had both the Val12 and Arg13 mutations in the same tumor and that one carcinoma had both the Arg12 and Cys12 mutations in the same tumor. The presence of two different mutant Ki-*ras* alleles within the same tumor has been detected in other studies (37,39). Overall, the observed frequency of Ki-*ras* mutations in BALB/c lung tumors was 33% (2/6) in hyperplasias, 58% (10/19) in adenomas and 73% (8/11) in carcinomas.

The inactivation of tumor suppressor genes, such as p16^{Ink4a}, has been shown to play an important role in tumorigenesis (20). Similar to what was found in lung tumors of [D2×B6D2F$_1$]F$_2$ mice (40), SSCP and subsequent sequence analyses showed the presence of two point mutations in exon 2 of p16^{Ink4a} in BALB/c mice (Figure 3). Tumor samples 7 and 19 both exhibited a GTA→ATA (Val51→Ile) transition. These G→A mutations were also found in lung (40) and liver tumors of transplacentally MC-treated mice (41) and suggest the possibility that endogenous aberrant methylation may have mediated damage to the p16^{Ink4a} gene. The codon number is

Table III. Frequency of Ki-*ras* mutations in adult and transplacentally treated mice

	BALB/c (this study)	[D2×B6D2F$_1$]F$_2$ (Leone-Kabler *et al.*, 1997)	A/J (Horio *et al.*, 1996)
Hyperplasias	33% (2/6)	60% (6/10)	43% (3/7)
Adenomas	58% (10/19)	80% (24/30)	59% (13/22)
Carcinomas	73% (8/11)	100% (7/7)	82% (9/11)

Frequency of Ki-*ras* mutations in the offspring of BALB/c and [D2×B6D2F$_1$]F$_2$ mice treated *in utero* with a single injection of MC on day 17 of gestation and adult A/J mice treated with a single injection of urethane at 6–10 weeks of age.

relative to the murine p16^{Ink4a} sequence reported by Gressani *et al.* (GenBank accession no. AF004588) and the rat p16^{Ink4a} sequence reported by Belinsky *et al.* (GenBank accession no. L81167), in which an additional GCC (Ala) triplet at codon 11 is present relative to the sequence reported by Quelle *et al.* (42). No mutations were observed by SSCP analyses in exon 1α of the p16^{Ink4a} gene or exon 1β of the p19^{ARF} gene. Previous studies by this and other laboratories (reviewed in ref. 43) have failed to identify mutations in *p53* in rodent lung tumors, hence, this gene was not screened in the present study.

Discussion

Our laboratory has been studying the interaction between environmental and genetic factors in the induction of lung tumors following *in utero* exposure to dietary and environmental carcinogens, as carcinogen susceptibility has been determined in the adult but not the developing organism. Utilizing 3-MC as a model carcinogen, we previously demonstrated that transplacentally treated [D2×B6D2F$_1$]F$_2$ mice develop lung and liver tumors. The present study sought to examine the carcinogenicity of transplacental exposure to MC in BALB/c mice and to compare these results with those previously reported in the more resistant [D2×B6D2F$_1$]F$_2$ mouse strain (24). In addition, we were interested in assessing the molecular pathogenesis of the chemically induced tumors, as well as the modulatory effects of BHT in BALB/c lung tumors.

In utero exposure to MC induced lung tumors in both the [D2×B6D2F$_1$]F$_2$ (24) and BALB/c (this study) mouse strains. The incidence and multiplicity of lung tumors induced by MC in BALB/c mice did not differ appreciably from that observed previously in [D2×B6D2F$_1$]F$_2$ mice. Similar to human lung neoplasms, lung tumors removed from BALB/c and [D2×B6D2F$_1$]F$_2$ mice showed a high incidence of Ki-*ras* mutations, 56 (20/36) and 79% (37/47), respectively. There was a predominance of guanine mutations which is consistent with the metabolism of MC to reactive electrophiles that bind mainly to guanine bases to form DNA adducts (44). Such adduct profiles are found in both fetal and adult tissues (13). An increase in the incidence of mutations in Ki-*ras* was observed in the progression of BALB/c and [D2×B6D2F$_1$]F$_2$ lung tumors from hyperplasias to adenomas to carcinomas (Table III). More specifically, in BALB/c and [D2×B6D2F$_1$]F$_2$ mice 73–100% of the carcinomas exhibited Ki-*ras* mutations compared with 33–60% of the hyperplasias. This trend has also been observed in carcinogen-treated adult mice. As shown in Table III, Horio *et al.* (45) found 43% (3/7) of urethane-induced lung hyperplasias in adult A/J mice to exhibit Ki-*ras* mutations and 82% (9/11) of late stage adenomas to exhibit mutations in Ki-*ras*. These data presented by our laboratory and others lend further support to the important role the Ki-*ras* gene plays at early stages of human and experimentally induced lung neoplasia (25,46–48). In addition, the results suggest that activation of Ki-*ras* alone cannot account for tumor susceptibility, for the frequency of Ki-*ras* mutations is remarkably similar in the relatively tumor-resistant [D2×B6D2F$_1$]F$_2$ strain and the more susceptible BALB/c and A/J strains. While Ki-*ras* is well recognized as a lung tumor susceptibility gene, our results provide additional evidence that other loci are associated with susceptibility to chemically induced lung tumors and/or that the Ki-*ras* polymorphism does not play an important role in tumor susceptibility in transplacentally induced neoplasia. The two strains did, however, differ in mutational spectrum, with the BALB/c lesions exhibiting a high incidence (62%) of G→C transversions and the [D2×B6D2F$_1$]F$_2$ lesions exhibiting a preponderance of G→T (84%) mutations. Whether the different mutant RAS proteins influence the oncogenic potential of the lung lesions is currently not known.

The Cys12 mutations observed in the neoplasms of both strains were present predominantly in the earlier stage tumors (hyperplasias), while the other mutations, Val12, Arg12, Arg13 and Arg61, were found only in the later stage neoplasms. Thus, Cys12 may have less oncogenic potential than the other early alterations. Similar findings have been found in adult mice. In a study by Nuzum *et al.* (49), urethane-induced lung adenomas in A/J mice primarily exhibited a leucine transversion at codon 61 of Ki-*ras*, while lung adenocarcinomas primarily exhibited an Arg transition at codon 61. These findings are important in that determining the types of mutations present in a tumor could provide prognostic data as to the potential of the tumor to progress to a more malignant phenotype. This could ultimately have diagnostic, as well as prognostic, value.

The percentage of BALB/c lung tumors with mutations in exon 2 of p16^{Ink4a} agreed well with the percentage found previously in [D2×B6D2F$_1$]F$_2$ mice (40), 6 and 7%, respectively. This relatively low incidence of p16^{Ink4a} mutations is consistent with that reported by Herzog *et al.* (50) in which frequent inactivation of p16^{Ink4a} was observed in mouse lung tumor cell lines (72%), while infrequent inactivation of p16^{Ink4a} was observed in primary mouse lung carcinomas (6%). All the mutations detected in either strain were G→A or C→T transitions. Recent studies by Herman *et al.* (51) and Swafford *et al.* (52) have suggested that methylation within p16^{Ink4a} may inactivate this gene during the early stages of tumorigenesis (53). Whether or not endogenous methylation-mediated damage is related to CpG sites or transcriptional silencing of the p16^{Ink4a} gene due to aberrant hypermethylation of the promoter region (54) remains a question. While the p16^{Ink4a} polymorphisms found in these different strains do not appear to be determinants of structural alterations in this gene, they may alter susceptibility of these mice to methylation. No mutations in exon 1β of p19^{ARF} were observed in lung tumor tissue excised from BALB/c or [D2×B6D2F$_1$]F$_2$ mice. p19^{ARF}, the alternative p16^{Ink4a} transcript, has been demonstrated to indirectly decrease cell proliferation by binding to the MDM2 protein, preventing its inhibition of *p53* (54–56). Despite its apparent importance in cell growth regulation, results from this and other studies (57) suggest that the p19^{ARF} gene may not be a primary target in murine lung tumorigenesis.

Although the tumor incidence in BALB/c mice did not differ significantly between mice treated or not treated with BHT, there was an interesting trend observed in mice co-treated with BHT. There was some evidence that BHT increased the number of tumors at the higher doses of MC. At the 15 mg/kg dose, a greater than doubling of tumor incidence and a quadrupling of tumor multiplicity were observed in BHT-treated mice. It is possible that mice exposed to BHT may have developed tumors earlier than mice exposed only to MC, but that by the time all the mice were killed the former group had also developed tumors. In contrast to reports suggesting that BHT may influence the frequency of Ki-*ras* mutations in adult lung tumors of mice (58), data from the present study show that BHT did not have any significant effect on the frequency of Ki-*ras* mutations in transplacentally treated BALB/c mice. However, the lack of a statistically significant effect of BHT may have possibly been due to the relatively low number of mice used in this study.

The susceptibility to pulmonary adenomas is associated with Ki-*ras* polymorphisms. The data in the present study suggest that this locus may not play an important role in lung tumor susceptibility in transplacentally induced lung neoplasia. The morphology of mouse lung tumors chemically induced in early fetal development is different from that in lung tumors in mice treated as adults (59), suggesting that the process of tumorigenesis in the fetus may involve different mechanisms than in the adult. This may explain why BHT did not affect the mutational spectrum of Ki-*ras*. In contrast to our findings, studies reported by Matzinger *et al.* (60) showed that in adult A/J mice 100% of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumors contained a mutation at codon 12 of Ki-*ras* while only 32% of NNK-induced and BHT-promoted tumors exhibited this mutation. It is possible that the promotional effects of BHT seen in adults were nullified due to the substantial growth that occurs neonatally.

These results and those of our previous study provide further evidence of the high sensitivity of the individual fetus to environmental toxicants. Overall, 21 of the 36 (58%) lung tumors of BALB/c mice and 37 of the 47 (79%) lung tumors of $[D2\times B6D2F_1]F_2$ mice exhibited mutations in Ki-*ras* and/or $p16^{Ink4a}$, demonstrating that these mice harbor some of the same types of genetic lesions observed in human tumors. These findings support this model as an appropriate paradigm for studying human cancer pathogenesis and provide evidence as to the importance of regulation of such potentially carcinogenic compounds during the sensitive prenatal period of development. Moreover, the findings suggest that mutagenic damage to the Ki-*ras* gene may be a critical early event mediating lung tumorigenesis in transplacentally MC-treated $[D2\times B6D2F_1]F_2$ and BALB/c mice. Differences observed in the Ki-*ras* mutational spectrum between the two strains may account, at least in part, for their differential susceptibility to lung tumor initiation. These results, combined with our previous studies, highlight the potential consequences of *in utero* exposure to environmental agents for the developing organism. Given the documented higher sensitivity of the fetus to chemical carcinogen exposure (1–3), this differential sensitivity should be taken into account when regulatory guidelines are established for particular chemicals. In addition, pregnant women who smoke should be concerned with the possibility that they may be placing their offspring at risk of cancer development later in adult life.

Acknowledgements

The authors would like to thank Elyse Jung for assistance with the automated sequencing and Hermina Tulli for the processing of tissue samples. This project was supported in part by grants ES08252 (M.S.M.), ESO6501 (M.S.M.), CA33497 (A.M.M.) and Cancer Center Support Grant CA12197, which provided support for the Analytical Imaging Core Facility, the DNA Synthesis Core Laboratory and the DNA Sequencing and Gene Analysis Facility.

References

1. Rice,J.M. (1979) Perinatal period and pregnancy: intervals of high risk for chemical carcinogens. *Environ. Health Perspect.*, **29**, 23–27.
2. Miller,M.S., Jones,A.B. and Anderson,L.M. (1990) The formation of 3-methylcholanthrene-initiated lung tumors correlates with induction of cytochrome P4501A1 by the carcinogen in fetal but not adult mice. *Toxicol. Appl. Pharmacol.*, **104**, 235–245.
3. Miller,M.S. (1994) Transplacental lung carcinogenesis: a pharmacogenetic mouse model for the modulatory role of cytochrome P4501A1 on lung cancer. *Chem. Res. Toxicol.*, **7**, 471–481.
4. Sipowicz,M.A., Amin,S., Desai,D., Kasprzak,K.S. and Anderson,L.M. (1997) Oxidative DNA damage in tissues of pregnant female mice and fetuses caused by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Cancer Lett.*, **117**, 87–91.
5. Perera,F.P., Whyatt,R.M., Jedrychowski,W., Rauh,V., Manchester,D., Santella,R.M. and Ottman,R. (1998) Recent developments in molecular epidemiology: a study of the effects of environmental polycyclic aromatic hydrocarbons on birth outcomes in Poland. *Am. J. Epidemiol.*, **147**, 309–314.
6. Greenberg,R.S. and Shuster,J.L.Jr (1985) Epidemiology of cancer in children. *Epidemiol. Rev.*, **7**, 22–48.
7. Zahm,S.H. and Devesa,S.S. (1995) Childhood cancer: overview of incidence trends and environmental carcinogens. *Environ. Health Perspect.*, **103** (suppl. 6), 177–184.
8. Conney,A.H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. *Cancer Res.*, **42**, 4875–4917.
9. Christianson,R.E. (1979) Gross differences observed in the placentas of smokers and nonsmokers. *Am. J. Epidemiol.*, **110**, 178–187.
10. Anderson,L.M., Jones,A.B., Miller,M.S. and Chauhan,D.P. (1989) Metabolism of transplacental carcinogens. In Napalkov,N.P., Rice,J.M., Tomatis,L. and Yamasaki,H. (eds) *Perinatal and Multigeneration Carcinogenesis.* IARC Scientific Publications no. 96, Lyon, France, pp. 155–188.
11. Manchester,D.K., Bowman,E.D., Parker,N.B., Caporaso,N.E and Weston,A. (1992) Determinants of polycyclic aromatic hydrocarbon–DNA adducts in human placenta. *Cancer Res.*, **52**, 1499–1503.
12. Whyatt,R.M., Santella,R., Garte,S.J., Ottman,R., Gladek-Yarborough,A., Young,T., Jedrychowski,W., Cosma,G. and Perera,F.P. (1995) Effects of environmental exposures on DNA damage in women and the developing fetus and its modulation by genetic/metabolic factors. *Proc. Am. Assoc. Cancer Res.*, **36**, 1690.
13. Lu,L.W., Harvey,R.G., Lee,H., Baxter,J.R. and Anderson,L.M. (1990) Age-, tissue-, and Ah genotype-dependent differences in the binding of 3-methylcholanthrene and its metabolite(s) to mouse DNA. *Cancer Res.*, **50**, 4239–4247.
14. Anderson,L.M., Jones,A.B., Riggs,C.W. and Ohshima,M. (1985) Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. *Carcinogenesis*, **6**, 1389–1393.
15. Miller,M.S., Juchau,M.R., Guengerich,F.P., Nebert,D.W. and Raucy,J.L. (1996) Symposium overview: drug metabolic enzymes in developmental toxicology. *Fundam. Appl. Toxicol.*, **34**, 165–175.
16. Rodenhuis,S. (1992) *ras* and human tumors. *Semin. Cancer Biol.*, **3**, 241–247.
17. Reynolds,S.H., Wiest,J.S., Devereux,T.R., Anderson,M.W. and You,M. (1992) Protooncogene activation in spontaneously occurring and chemically induced rodent and human lung tumors. In Klein-Szanto,A.J.P., Anderson,M.W., Barrett,J.C. and Slaga,T.J. (eds) *Comparative Molecular Carcinogenesis.* Wiley-Liss, New York, NY, pp. 303–320.
18. Mills,N.E., Fishman,C.L., Rom,W.N., Dubin,N. and Jacobson,D.R. (1995) Increased prevalence of K-*ras* oncogene mutations in lung adenocarcinoma. *Cancer Res.*, **55**, 1444–1447.
19. Kamb,A. (1995) Cell-cycle regulators and cancer. *Trends Genet.*, **11**, 136–140.
20. Foulkes,W.D., Flanders,T.Y., Pollock,P.M. and Hayward,N.K. (1997) The CDKN2A (*p16*) gene and human cancer. *Mol. Med.*, **3**, 5–20.

21. Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323- 331.
22. Sherr,C.J. (1996) Cancer cell cycles. *Science*, **274**, 1672–1677.
23. Serrano,M. (1997) Minireview. The tumor suppressor protein $p16^{INK4a}$. *Exp. Cell Res.*, **237**, 7–13.
24. Wessner,L.L., Fan,M., Schaeffer,D.O., McEntee,M.F. and Miller,M.S. (1996) Mouse lung tumors exhibit specific Ki-*ras* mutations following transplacental exposure to 3-methylcholanthrene. *Carcinogenesis*, **17**, 1519–1526.
25. Malkinson,A.M. (1989) The genetic basis of susceptibility to lung tumors in mice. *Toxicology*, **54**, 241–271.
26. Ryan,J., Barker,P.E., Nesbitt,M.N. and Ruddle,F.H. (1987) *KRAS2* as a genetic marker for lung tumor susceptibility in inbred mice. *J. Natl Cancer Inst.*, **79**, 1351–1357.
27. Herzog,C.R. and You,M. (1997) Sequence variation and chromosomal mapping of the murine *Cdkn2a* tumor suppressor gene. *Mamm. Genome*, **8**, 65–66.
28. Malkinson,A.M., Koski,K.M., Evans,W.A. and Festing,M.F.W. (1997) Butylated hydroxytoluene exposure is necessary to induce lung tumors in BALB mice treated with 3-methylcholanthrene. *Cancer Res.*, **57**, 2832–2834.
29. Witschi,H. (1993) Modulation of tumor development by butylated hydroxytoluene in experimental animals (Review). *Toxicol. Ind. Health*, **9**, 259–281.
30. Witschi,H., Malkinson,A.M. and Thompson,J.A. (1989) Metabolism and pulmonary toxicity of butylated hydroxytoluene (BHT). *Pharmacol. Ther.*, **42**, 89–113.
31. Miller,A.C.K., Dwyer,L.D., Auerbach,C.E., Miley,F.B., Dinsdale,D. and Malkinson,A.M. (1994) Strain-related differences in the pneumotoxic effects of chronically administered butylated hydroxytoluene on protein kinase C and calpain. *Toxicology*, **90**, 141–159.
32. Guan,X., Hardenbrook,J., Fernstrom,M.J., Chaudhuri,R., Malkinson,A.M. and Ruch,R.J. (1995) Down-regulation by butylated hydroxytoluene of the number and function of gap junctions in epithelial cell lines derived from mouse lung and rat liver. *Carcinogenesis*, **16**, 2575–2582.
33. Dwyer-Nield,L.D., Thompson,J.A., Peljak,G., Squier,M.K., Barker,T.D., Parkinson,A., Cohen,J.J., Dinsdale,D. and Malkinson,A.M. (1998) Selective induction of apoptosis in mouse and human lung epithelial cell lines by the tert-butyl hydroxylated metabolite of butylated hydroxytoluene: a proposed role in tumor promotion. *Toxicology*, **130**, 115–127.
34. Faccini,J.M., Abbott,D.P. and Paulus,G.J.J. (1990) *Mouse Histopathology: a Glossary for Use in Toxicity and Carcinogenicity Studies*. Elsevier Scientific, New York, NY, pp. 48–62.
35. Wright,D.K. and Manos,M.M. (1990) Sample preparation from paraffin-embedded tissues. In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds) *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA, pp. 153–158.
36. Miller,M.S., Baxter,J.L., Moore,J.W., Lewis,J.D. and Schuller,H.M. (1994) Molecular characterization of neuroendocrine lung tumors induced in hamsters by treatment with nitrosamines and hyperoxia. *Int. J. Oncol.*, **4**, 5–12.
37. Leone-Kabler,S., Wessner,L.L., McEntee,M.F., D'Agostino,R.B.Jr and Miller,M.S. (1997) Ki-*ras* mutations are an early event and correlate with tumor stage in transplacentally-induced murine lung tumors. *Carcinogenesis*, **18**, 1163–1168.
38. You,M., Candrian,U., Maronpot,R.R., Stoner,G.D. and Anderson,M.W. (1989) Activation of the Ki-*ras* protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. *Proc. Natl Acad. Sci. USA*, **86**, 3070–3074.
39. Massey,T.E., Devereux,T.R., Maronpot,R.R., Foley,J.E. and Anderson, M.W. (1995) High frequency of K-*ras* mutations in spontaneous and vinyl chloride-induced lung tumors of relatively resistant B6C3F1 (C57BL/6J×BALB/cJ) mice. *Carcinogenesis*, **16**, 1065–1069.
40. Rollins,L.A., Leone-Kabler,S., O'Sullivan,M.G. and Miller,M.S. (1998) Role of tumor suppressor genes in transplacental lung carcinogenesis. *Mol. Carcinog.*, **21**, 177–184.
41. Gressani,K.M., Rollins,L.A., Leone-Kabler,S., Cline,J.M. and Miller,M.S. (1998) Induction of mutations in Ki-*ras* and *INK4a* in liver tumors of mice exposed *in utero* to 3-methylcholanthrene. *Carcinogenesis*, **19**, 1045–1052.
42. Quelle,D.E., Ashmun,R.A., Hannon,G.J., Rehberger,P.A., Trono,D., Richter,K.H., Walker,C., Beach,D., Sherr,C.J. and Serrano,M. (1995) Cloning and characterization of murine $p16^{INK4a}$ and $p15^{INK4b}$ genes. *Oncogene*, **11**, 635–645.
43. Miller,M.S. (1999) Tumor suppressor genes in rodent lung carcinogenesis—mutation of p53 does not appear to be an early lesion in lung tumor pathogenesis. *Toxicol. Appl. Pharmacol.*, **156**, 70–77.
44. Osborne,M.R., Brookes,P., Lee,H. and Harvey,R.G. (1986) The reaction of a 3-methylcholanthrene diol epoxide with DNA in relation to the binding of 3-methylcholanthrene to the DNA of mammalian cells. *Carcinogenesis*, **7**, 1345–1350.
45. Horio,Y., Chen,A., Rice,P., Roth,J.A., Malkinson,A.M. and Schrump,D.S. (1996) Ki-*ras* and *p53* mutations are early and late events, respectively, in urethane-induced pulmonary carcinogenesis in A/J mice. *Mol. Carcinog.*, **17**, 217–223.
46. Cazorla,M., Hernandez,L., Fernandez,P.L. *et al*. (1998) Ki-*ras* gene mutations and absence of *p53* gene mutations in spontaneous and urethane-induced early lung lesions in CBA/J mice. *Mol. Carcinog.*, **21**, 251–260.
47. Li,Z.-H., Zheng,J., Weiss,L.M. and Shibata,D. (1994) c-*K-ras* and *p53* mutations occur very early in adenocarcinoma of the lung. *Am. J. Pathol.*, **144**, 303–309.
48. Miller,M.S., Kabler,S.L., Rollins,L.A., Wessner,L.L., Fan,M., Schaeffer, D.O., McEntee,M.F. and O'Sullivan,M.G. (1998) Molecular pathogenesis of transplacentally induced mouse lung tumors. *Exp. Lung Res.*, **24**, 557–577.
49. Nuzum,E.O., Malkinson,AM. and Beer,D.G. (1990) Specific Ki-*ras* codon 61 mutations may determine the development of urethan-induced mouse lung adenomas or adenocarcinomas. *Mol. Carcinog.*, **3**, 287–295.
50. Herzog, C.R., Soloff,E.V., McDoniels,A.L., Tyson,F.L., Malkinson,A.M., Haugen-Strano,A., Wiseman,R.W., Anderson,M.W. and You,M. (1996) Homozygous codeletion and differential decreased expression of $p15^{INK4b}$, $p16^{INK4a}$-α and $p16^{INK4a}$-β in mouse lung tumor cells. *Oncogene*, **13**, 1885–1891.
51. Herman,J.G., Merlo,A., Mao,L., Lapidus,R.G., Issa,J.P., Davidson,N.E., Sidransky,D. and Baylin,S.B. (1995) Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.*, **55**, 4525–4530.
52. Swafford,D.S., Middleton,S.K., Palmisano,W.A., Nikula,K.J., Tesfaigzi,J., Baylin,S.B., Herman,J.G. and Belinsky,S.A. (1997) Frequent aberrant methylation of p16INK4a in primary rat lung tumors. *Mol. Cell. Biol.*, **17**, 1366–1374.
53. Belinsky,S.A., Nikula,K.J., Palmisano,W.A., Michels,R., Saccomanno,G., Gabrielson,E., Baylin,S.B and Herman,J.G. (1998) Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl Acad. Sci. USA*, **95**, 11891–11896.
54. Pomerantz,J., Schreiber-Agus,N., Liegeois,N.J. *et al*. (1998) The *Ink4a* tumor suppressor gene product, $p19^{Arf}$, interacts with MDM2 and neutralizes MDM2′s inhibition of p53. *Cell*, **92**, 713–723.
55. Stott,F.J., Bates,S., James,M.C. *et al*. (1998) The alternative product from the human *CDKN2A* locus, $p19^{ARF}$, participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.*, **17**, 5001–5014.
56. Kamijo,T., Zindy,F., Roussel,M.F., Quelle,D.E., Downing,J.R., Ashmun, R.A., Grosveld,G. and Sherr,C.J. (1997) Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product $p19^{ARF}$. *Cell*, **91**, 649–659.
57. Liggett,W.H. and Sidransky,D. (1998) Role of the *p16* tumor suppressor gene in cancer. *J. Clin. Oncol.*, **16**, 1197–1206.
58. Wang,X. and Witschi,H. (1995) Mutations of the Ki-*ras* protooncogene in 3-methylcholanthrene and urethane-induced and butylated hydroxytoluene-promoted lung tumors of strain A/J and SWR mice. *Cancer Lett.*, **91**, 33–39.
59. Branstetter,D.G., Stoner,G.D., Budd,C., Conran,P.B. and Goldblatt,P.J. (1988) Effect of gestational development on lung tumor size and morphology in the mouse. *Cancer Res.*, **48**, 379–386.
60. Matzinger,S.A., Gunning,W.T., You,M. and Castonguay,A. (1994) Ki-*ras* mutations in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-initiated and butylated hydroxytoluene-promoted lung tumors in A/J mice. *Mol. Carcinog.*, **11**, 42–48.

Received March 29, 1999; revised June 8, 1999; accepted June 25, 1999